REMARKS

Claims 1, 3-4, 8-27, 55, 57-58, 62-81, and 109-110 are pending after entry of the amendments set forth herein. With this Reply and Amendment, Claims 1, 3, 8-13, 16-18, 21-27, 55, 57, 62-67, 70-72, 75-81, 109-110 have been amended, Claims 2 and 56 have been canceled without prejudice. Support for the amendments is found in the specification and claims as originally filed. No new matter has been added.

Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Claim Objections

Item B

Claims 3 and 57 were objected to in view of a syntax error. Claims 3 and 57 have been amended to address the error.

Item C

Claims 21-27 and 75-81 were objected to because the term "dose response" should be hyphenated. Claims 21-27 and 75-81 have been amended to address the error.

Item D

Claims 24 and 78 were objected to in view of a syntax error. Claims 3 and 57 have been amended to address the error.

In view of the amendments to the claims, the Applicants respectfully request that the objections be withdrawn.

Claim Rejection Under 35 U.S.C. § 112, First paragraph (Enablement)

Claims 1-4, 8-14, 16-27, 55-58, 62-68, 70-81, and 109-110 are rejected under 35 U.S.C. § 112, first paragraph, allegedly because the specification, while being enabling for a method of identifying a agonist using a G protein coupled receptor (GPCR) and arrestin, does not reasonably provide enablement

for a method of identifying agonist using a transmembrane receptor (TMR), a biologically active fragment of a TMR or where the cell further comprises biologically active fragments of arrestin. In view of the amendments to the claims and the remarks made below, this rejection is respectfully traversed.

As the Applicants understand it, the rejection is based on an alleged lack of enablement for the following elements:

(a) all transmembrane receptors (TMRs);

(a) biologically active fragments of TMRs; and

(c) biologically active fragments of arrestin.

Each element of the rejection is addressed individually in greater detail below.

(a) all transmembrane receptors (TMRs)

The Office Action notes that the specification, as well as the art, is enabling for GPCRs. Therefore, in the spirit of expediting prosecution and without conceding to the correctness of the rejection, the claims have been amended to recite G coupled protein receptors (GPCRs).

(a) biologically active fragments of TMRs

As noted above, the claims have been amended to recite GPCR. With respect to the "biologically active fragments", the claims have been amended to recite "a modified GPCR". A modified GPCR is specifically defined in the specification on page 8, paragraph [0037]. In addition, the specification also provides examples of modified GPCRs, including those described in U.S. Patent Application Serial Numbers 09/993,844 (now U.S. Patent No. 7,018,812) and 10/054,616 (U.S. Published Patent Application No. 2003/0049643) (Specification, page 8, paragraph [0037]).

Furthermore, as suggested in the Office Action, the claims have also been amended to further define the biological activity of the modified GPCR. Specifically, Claims 1 and 55 have been amended to recite "a modified GPCR capable of activating intracellular signaling".

(c) biologically active fragments of arrestin.

As suggested in the office Action, the claims have also been amended to further define the biological activity. Claims 1 and 55 have been amended to recite "a fragment thereof capable of binding

a GPCR". Examples of arrestin fragments that are capable of binding a GPCR include the β-arrestin 318-419 fragment (Exhibit A: Pierce et al., PNAS 97(4):1489-1494 (2000)) and the β-arrestin 1-393 truncated form (Exhibit B: Kovoor et al., JBC 274(11)6831-6834 (1999)).

As such, the Applicants respectfully request that the rejection of Claims 1-4, 8-14, 16-27, 55-58, 62-68, 70-81, and 109-110 be withdrawn.

Claim Rejection Under 35 U.S.C. § 112, First paragraph (Written Description)

Claims 1-4, 8-14, 16-27, 55-58, 62-68, 70-81, and 109-110 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Applicants respectfully traverse.

As noted above, in the spirit of expediting prosecution and without conceding as to the correctness of the rejection, the claims have been amended to recite GPCR. The claims have also been amended to further define the biological activity of the modified GPCRs and fragments of arrestins. Moreover, the Applicants also provide herein examples of modified GPCRs that are capable of activating intracellular signaling (including U.S. Patent Application Serial Numbers 09/993,844 (now U.S. Patent No. 7,018,812) and 10/054,616 (U.S. Published Patent Application No. 2003/0049643) and arrestin fragments that are capable of binding a GPCR (Exhibits A and B).

Therefore, the pending Claims are in compliance with the written description requirement of 35 U.S.C. § 112, first paragraph. In view of the foregoing, Applicants respectfully request that the rejections of Claims 1-4, 8-14, 16-27, 55-58, 62-68, 70-81, and 109-110 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Claim Rejection Under 35 U.S.C. § 112 (second paragraph)

The Office Action has rejected several claims under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite and failing to distinctly claim the subject matter regarded as the invention. Each item of the rejection is addressed in detail below.

Item B

The Office Action has maintained the rejection of Claims 1 and 55. In maintaining the rejection the Office Action asserts that it would not be apparent to an artisan what constitutes a "biologically active" fragment of a TMR or an arrestin. As noted above, Claims 1 and 55 have been amended to remove the objectionable language and to recite "GPCR or a modified GPCR and an arrestin, or a fragment thereof capable of binding a GPCR".

Item C

Claims 1-4, 8-14, 16-27, 55-58, 62-28, 70-81, 109, and 110 stand rejected because the phrase "and wherein the signaling is activated as compared to" is allegedly unclear. Claim 1 and 55 have been amended for clarity to recite "and wherein GPCR signaling is activated in the presence of the test compound as compared to GPCR signaling in the absence of the test compound" as suggested in the Office Action.

Item D

Claims 1-4, 8-14, 16-27, 55-58, 62-28, 70-81, 109, and 110 stand rejected for allegedly omitting an essential element. Claims 1 and 55 have been amended for clarity to recite "wherein a reduction in GPCR internalization in the presence of the test compound as compared to the control compound indicates the test compound is a GPCR agonist capable of activating GPCR signaling while exhibiting reduced GPCR internalization". Moreover, Claims 1 and 55 have also been amended to provide the goal of the method in the conclusion step.

Item E

Claims 2, 10, 11, 56, 64, and 65 have been rejected for failing to spell out the acronym "GPCR" upon first use. Claims 1 and 55 have been amended to recite "G protein coupled receptor (GPCR)".

Item F

Claims 3, 9, 12, 13, 21, 22, 24, 26, 27, 57, 63, 66, 67, 75, 76, 78, 80, and 81 have been rejected for providing insufficient antecedent basis in the claims. Claims 1 and 55 have been amended for clarity to recite "measuring internalization of the GPCR" in element (d). In addition, dependent claims 3, 9, 12, 13, 21, 22, 24, 26, 27, 57, 63, 66, 67, 75, 76, 78, 80, and 81 have been amended to recite "the internalization".

Item G

Claims 9 and 63 have been rejected for missing an essential element. Claims 9 and 63 have been amended to recite "determining the localization of the GPCR"

Item H

Claims 17, 18, 71, and 72 have been rejected for alleged lack of clarity and omitting an essential element. Claims 17 and 71 have been amended for clarity to recite "wherein the steps (a) – (f) are repeated, and wherein the GPCR used in the repeated steps is from a different species than the GPCR used in steps (a) – (f)". In addition, Claims 18 and 72 have also been amended for clarity to recite "wherein a test compound that is used in steps (a) – (f) is not used in the repeated steps".

Item I

Claims 24, 25, 78, and 79 have been rejected for clarity because the terms "reduced" and "greater" are allegedly arbitrary. Applicants respectfully disagree.

MPEP §2173.05(b) notes that "[t]he fact that claim language, including terms of degree, may not be precise, does not automatically render the claim indefinite under 35 U.S.C. 112, second paragraph". The MPEP further notes that if a relative term is used it must be determined whether a standard is disclosed or whether one of ordinary skill in the art would be apprised of the scope of the claim.

Claims 24, 25, 78, and 79 all provide a reference point, or standard, to which the relative term can be compared. For example, amended Claims 24 and 78 recite that the "dose-response curve for the internalization measurement in the presence of the test compound **is less than** the dose-response curve for the internalization measurement in the presence of the control compound". Therefore, the claims

specifically provide a reference point for determining "less than" as being compared to the measurement in the presence of the control compound. In addition, Claims 25 and 79 recite that the "dose-response curve for the signaling measurement in the presence of the test compound is approximately equal to or greater than the dose-response curve for the signaling measurement in the presence of the control compound". Therefore, the claims specifically provide a reference point for determining "greater than" as being compared to the measurement in the presence of the control compound.

Since the claims include a standard within the claims that defines the reference point for the relative term, the claims cannot be ambiguous and lack clarity. Therefore, the applicants respectfully request that this rejection be withdrawn.

Item J

Claims 55-58, 62-81, and 110 have been rejected allegedly being unclear as to which "compound" steps (c) and (d) refer. Claim 55 has been amended in steps (c) and (d) to recite "test compound" in order to clarify the specific compound.

Accordingly, in view of the amendments to the claims and the remarks made herein, the Applicants respectfully request that these rejections be withdrawn.

Claim Rejection Under 35 U.S.C. § 102

Claims 1-3, 5, 9, 11-13, 16, 20, 55-59, 63-67, 70, and 74 are allegedly rejected under 35 U.S.C. § 102(b) as being anticipated by Barak *et al.* (US Patent 6,110,693). In view of the amendments to the claims and remarks made herein, this rejection is respectfully traversed.

"A claim is anticipated only if *each and every element* as set forth in the claim is found, either expressly or inherently described, *in a single prior art reference*." *Verdegaal Bros. v. Union Oil Co. of California* 2 USPQ2d 1051, 1053 (Fed. Cir. 1987), emphasis added. See also, MPEP § 2131.

Amended Claims 1 and 55, recite in part, methods of identifying G coupled receptor (GPCR) agonist, wherein the GPCR agonist is capable of activating GPCR signaling while exhibiting reduced

GPCR internalization as compared to a control compound. As such, the method requires measurement of GPCR internalization and measurement of GPCR signaling at two or more time points (Claim 1) or GPCR signaling at one or more concentrations of the test compound (Claim 55).

In contrast, Barak *et al.* is directed in part to a method of screening a test compound for GPCR agonist activity, with a cell expressing a GPCR and an arrestin detectable molecule conjugate, exposing the cell to a test compound, and detecting movement of the detectable molecule to the membrane edge after exposure of the cell to the test compound. The cited reference does not teach measurement of both (1) GPCR internalization and (2) GPCR signaling at various time points or concentrations of test compound in order to identify a GPCR agonist capable of activating GPCR signaling while exhibiting reduced GPCR internalization.

Accordingly, since Barak *et al.* fails to teach each and every limitation found in the claims, the cited reference cannot anticipate the claims. Therefore, the Applicants respectfully request that this rejection be withdrawn.

Claim Rejection Under 35 U.S.C. §103

Claims 1-4, 8-14, 16-27, 55-58, 62-68, 70-81, and 109-110 have been rejected under 35 U.S.C. § 103(a) for allegedly being unpatentable over Barak *et al.* (U.S. Patent 6,110,693) in view of Knudsen, *et al.* (WO0246763). In view of the amendments to the claims and remarks made herein, this rejection is respectfully traversed.

To establish a *prima facie* case, three basic criteria must be met: (1) the prior art must provide one of ordinary skill with a suggestion or motivation to modify or combine the teachings of the references relied upon by the Patent Office to arrive at the claimed invention; (2) the prior art must provide one of ordinary skill with a reasonable expectation of success; and (3) the prior art, either alone or in combination, must teach or suggest each and every limitation of the rejected claims. The motivation to modify and/or combine references and the reasonable expectation of success, must come from the prior art, not Applicants' disclosure. *In re Vaeck* 20 USPQ2d 1438 (Fed. Cir. 1991). See also MPEP § 2142.

The present invention is directed to methods of identifying a GPCR agonist capable of activating GPCR signaling while exhibiting reduced GPCR internalization as compared to a control compound. As such, the method requires measurement of GPCR internalization and measurement of GPCR signaling at two or more time points (Claim 1) or GPCR signaling at one or more concentrations of the test compound (Claim 55). Therefore, both of (1) GPCR internalization and (2) GPCR signaling over a period of time or at various concentrations of the test compound lead to the proper identification of a GPCR agonist by the recited method.

As noted above Barak *et al.* fails to teach measurement of both (1) GPCR internalization and (2) GPCR signaling over a period of time or at various concentrations of the test compound. Knudsen, *et al.* has been cited for teaching a method of detecting the level of GPCR bound arrestin to screen chemical libraries, such as combinatorial chemical libraries, including chemical compounds that have been synthesized from a synthetic series of reactions. As such, Knudsen, *et al.* also fails to teach the combined measurement of GPCR internalization and GPCR signaling.

Based on the individual or combined teaching of Barak *et al.* and Knudsen, *et al.*, one of skill in the art would only be able to identify an agonist of GPCR that promotes binding of arrestin to the GPCR. The combined teaching of the references would not identify a GPCR agonist capable of activating GPCR signaling while also exhibiting reduced GPCR internalization as compared to a control compound. As described in greater detail in the present application, internalized GPCRs are not responsive to agonists or ligands, resulting in an attenuation of the signaling ability of the GPCR. Therefore, both of (1) GPCR internalization and (2) GPCR signaling lead to the proper identification of a GPCR agonist by the recited method.

As described above, Barak *et al.* fails to teach every element of the claimed methods. The secondary reference, Knudsen *et al*, does not cure the deficiencies of Barak *et al.* Therefore, none of the cited references, individually or in combination, teach or suggests the instant invention. Therefore, the Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

Applicants respectfully submit that the claims are now in condition for allowance and early notification to that effect is respectfully requested. If the Examiner feels there are further unresolved issues, the Examiner is respectfully requested to phone the undersigned at (415) 442-1000.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

Dated: April 3, 2007 By: _____

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Enclosures:

- Exhibit A: Pierce et al., PNAS 97(4):1489-1494 (2000).
- Exhibit B: Kovoor et al., JBC 274(11)6831-6834 (1999).

1-SF/7514649.1

Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors

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Contributed by Robert J. Lefkowitz, December 10, 1999

Acting through a number of distinct pathways, many G protein-coupled receptors (GPCRs) activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. Recently, it has been shown that in some cases, clathrin-mediated endocytosis is required for GPCR activation of the ERK/MAPK cascade, whereas in others it is not. Accordingly, we compared ERK activation mediated by a GPCR that does not undergo agonist-stimulated endocytosis, the α_{2A} adrenergic receptor (α_{2A} AR), with ERK activation mediated by the β_2 adrenergic receptor (β_2 AR), which is endocytosed. Surprisingly, we found that in COS-7 cells, ERK activation by the α_{2A} AR, like that mediated by both the β_2 AR and the epidermal growth factor receptor (EGFR), is sensitive to mechanistically distinct inhibitors of clathrin-mediated endocytosis, including monodansylcadaverine, a mutant dynamin I, and a mutant β-arrestin 1. Moreover, we determined that, as has been shown for many other GPCRs, both α_{2A} and β_2 AR-mediated ERK activation involves transactivation of the EGFR. Using confocal immunofluorescence microscopy, we found that stimulation of the β_2 AR, the α_{2A} AR, or the EGFR each results in internalization of a green fluorescent protein-tagged EGFR. Although β_2 AR stimulation leads to redistribution of both the β_2 AR and EGFR, activation of the α_{2A} AR leads to redistribution of the EGFR but the α_{2A} AR remains on the plasma membrane. These findings separate GPCR endocytosis from the requirement for clathrin-mediated endocytosis in EGFR transactivation-mediated ERK activation and suggest that it is the receptor tyrosine kinase or another downstream effector that must engage the endocytic machinery.

Many G protein-coupled receptors (GPCRs) have been shown to activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. Recently, it was discovered that for some GPCRs, events associated with the termination of receptor signaling are also involved in signaling to the MAPK cascade (1). Receptor phosphorylation, β-arrestin recruitment, and clathrin-mediated endocytosis have all been implicated in GPCR-mediated MAPK activation. Among GPCRs, the involvement of clathrin-mediated endocytosis in MAPK activation was first established for the β_2 adrenergic receptor (β_2 AR) (2). Specifically, dominant-negative forms of dynamin I and of β-arrestin 1, which inhibit clathrin-mediated endocytosis of the β_2 AR, also block isoproterenol-stimulated MAPK activation.

Additional studies addressing the role of clathrin-mediated endocytosis in GPCR-mediated ERK activation have yielded often conflicting results. In addition to the β_2 AR, our laboratory has reported that blocking receptor endocytosis attenuates MAPK signaling by endogenously expressed lysophosphatidic acid (LPA), thrombin, and bombesin receptors in Rat-1 fibroblasts (3) and by the 5-HT_{1A} receptor expressed in 293 cells (4). MAPK activation by the m1 muscarinic receptor (5), and the μ, δ, and κ opioid receptors (6, 7) are also reported to be sensitive to inhibitors of endocytosis, whereas MAPK signaling by the α_{2A}, α_{2B}, and α_{2C} adrenergic receptors (8, 9), the CB1 cannabinoid receptor (10), the m3 muscarinic receptor (11), the CXCR2 (12), the κ opioid receptor (13), and the B2 bradykinin receptor (14) have been shown to be independent of GPCR endocytosis. In some cases, notably the κ opioid receptor, MAPK signaling has been shown to be both sensitive and insensitive to inhibitors of clathrin-mediated endocytosis (6, 13).

One major pathway of GPCR-mediated MAPK activation converges with the pathway used by many receptor tyrosine kinases (RTKs) (15) (Scheme 1). This pathway, known as RTK "transactivation," has been demonstrated for many GPCRs, including the LPA receptor, the thrombin receptor, and the endothelin receptor (16–18). In this pathway, GPCR stimulation leads to the release of $G_{\beta\gamma}$ subunits, which, through unknown effectors, leads to activation and tyrosine phosphorylation of RTKs, such as the epidermal growth factor receptor (EGFR) (16). Subsequent to RTK phosphorylation, the steps involved in GPCR-mediated and RTK-mediated ERK activation are indistinguishable (Scheme 1) (19, 20).

$$\text{GPCR} \rightarrow G_{\beta\gamma} \rightarrow \rightarrow \text{RTK} \rightarrow \text{Shc} \rightarrow \text{Grb2-mSOS} \rightarrow \text{Ras} \rightarrow \text{Raf} \rightarrow \text{MEK} \rightarrow \text{MAPK}$$

Scheme 1.

Because direct EGF-induced ERK activation has been shown to depend on clathrin-mediated endocytosis (21), we hypothesized that the sensitivity of GPCR-mediated ERK activation might correlate with signaling via EGFR transactivation. That is, we hypothesized that, in cells in which GPCRs activate MAPK via transactivation of the EGFR, ERK activation would be sensitive to inhibitors of endocytosis, regardless of whether the GPCR itself underwent agonist-induced internalization. To test this hypothesis, we have examined the role of clathrin-mediated endocytosis in ERK activation via internalizing β_2 ARs and noninternalizing α_{2A} ARs in COS-7 cells, a cell type in which both receptors stimulate MAPK primarily via EGFR transactivation.

Abbreviations: RTK, receptor tyrosine kinase; LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; AR, adrenergic receptor; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; MDC, monodansylcadaverine; HA, hemagglutinin; GFP, green fluorescent protein.

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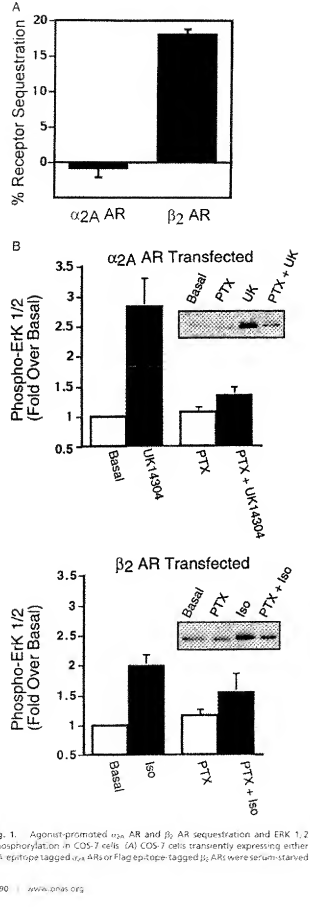

Fig. 1. Agonist-promoted α_{2A} AR and β_2 AR sequestration and ERK 1,2 phosphorylation in COS-7 cells. (*A*) COS-7 cells transiently expressing either HA epitope tagged α_{2A} ARs or Flag epitope-tagged β_2 ARs were serum-starved overnight and exposed to UK14304 (10 μM) or isoproterenol (10 μM), respectively, for 30 min at 37°C. Cell-surface receptors were labeled with a 12CA5 monoclonal antibody (Roche) or an M2 Flag monoclonal antibody (Sigma) by using FITC-conjugated goat anti-mouse IgG as a secondary antibody. Receptor sequestration was quantified as the loss of cell-surface receptors in agonist-treated cells, measured by using flow cytometry. The data are expressed as the mean ± SEM of four independent experiments performed in triplicate. (*B*) Appropriately transfected COS-7 cells were serum-starved overnight in the presence or absence of pertussis toxin (100 ng/ml) before stimulation with 1 μM UK14304 (*Upper*) or 1 μM isoproterenol (*Lower*) for 5 min. Aliquots of whole-cell lysate (approximately 30 μg of protein per lane) were resolved by SDS·PAGE, and ERK 1,2 phosphorylation was detected by protein immunoblotting by using rabbit polyclonal phospho-MAPK specific IgG. Data are expressed as the fold ERK 1,2 phosphorylation over the basal value in appropriately transfected cells. The data are expressed as the mean ± SEM of three independent experiments.

Materials and Methods

Materials. Tyrphostin AG1478 and recombinant EGF were from Calbiochem, monodansylcadaverine (MDC) was from Sigma, and pertussis toxin was from List Biological Laboratories (Campbell, CA). Anti-phospho-MAPK antibodies were from New England Biolabs, the total ERK 1/2 antibody and the EGFR antibodies were from Upstate Biotechnology (Lake Placid, NY), and the anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY). The unlabeled and rhodamine-labeled 12CA5 antibodies were from Roche Biochemicals, and the M2 Flag antibody was from Sigma. The anti-hemagglutinin (HA) affinity beads were from Covance (Princeton, NJ). Secondary antibodies were from Jackson ImmunoResearch (Indianapolis). All other reagents were standard laboratory grade.

Plasmids. HA-α_{2A} was obtained from Brian Kobilka (Stanford Univ.), β-arrestin 1 318–419 from J. L. Benovic (Thomas Jefferson Univ.), EGFR–green fluorescent protein (GFP) from A. Sorkin (University of Colorado Health Sciences Center), and HA-ERK-1 from J. Pouyssegur (Univ. of Nice). All other plasmids were constructed in our laboratory.

Tissue Culture. COS-7 cells were maintained in DMEM containing 10% fetal bovine serum and 100 μg/ml gentamicin. HEK293 cells were maintained in modified Eagle's medium containing 10% fetal bovine serum and 100 μg/ml gentamicin. Cells were transiently transfected by using Lipofectamine as described (16). Experiments were performed 2–3 days posttransfection, and in all cases, cells were serum starved overnight in medium containing 10 mM Hepes, 0.1% BSA, and 100 μg/ml gentamicin.

Sequestration Assays. COS-7 cells transiently expressing HA epitope-tagged α_{2A} ARs or Flag epitope-tagged β_2 ARs were exposed to isoproterenol (10 μM) or UK14304 (10 μM), respectively, for 30 min at 37°C. Cell-surface receptors were labeled with a 12CA5 monoclonal antibody (Roche) or an M2 Flag monoclonal antibody (Sigma) by using FITC-conjugated goat anti-mouse IgG as a secondary antibody. Receptor sequestration was quantified as loss of cell-surface receptors in agonist-treated cells measured by flow cytometry (22).

Immunoprecipitation. Serum-starved transfected cells were exposed to agonist at 37°C, washed once with ice-cold phosphate-buffered saline, lysed in glycerol lysis buffer [5 mM Hepes, 250 mM NaCl, 10% (vol/vol) glycerol, 0.5% Nonidet P-40, 2 mM EDTA, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin], clarified by centrifugation, and immunoprecipitated by using the appropriate antibodies. HA–ERK-1 was immunoprecipitated by using 20 μl of

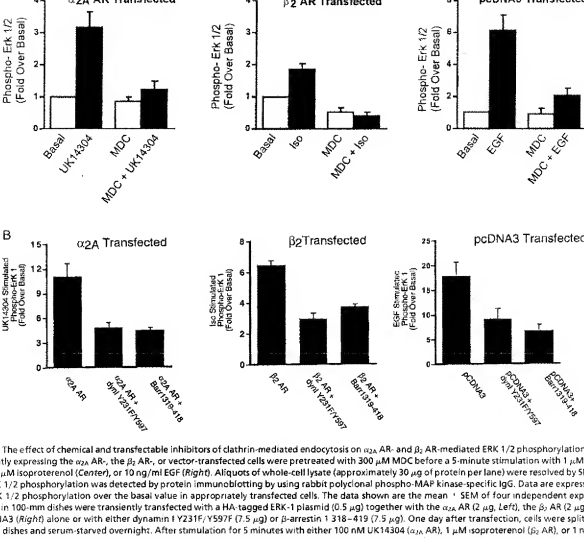

Fig. 2. The effect of chemical and transfectable inhibitors of clathrin-mediated endocytosis on α_{2A} AR- and β_2 AR-mediated ERK 1/2 phosphorylation. (*A*) Cells transiently expressing the α_{2A} AR-, the β_2 AR-, or vector-transfected cells were pretreated with 300 μM MDC before a 5-minute stimulation with 1 μM UK14304 (*Left*), 1 μM isoproterenol (*Center*), or 10 ng/ml EGF (*Right*). Aliquots of whole-cell lysate (approximately 30 μg of protein per lane) were resolved by SDS/PAGE, and ERK 1/2 phosphorylation was detected by protein immunoblotting by using rabbit polyclonal phospho-MAP kinase-specific IgG. Data are expressed as the fold ERK 1/2 phosphorylation over the basal value in appropriately transfected cells. The data shown are the mean ± SEM of four independent experiments. (*B*) Cells in 100-mm dishes were transiently transfected with a HA-tagged ERK-1 plasmid (0.5 μg) together with the α_{2A} AR (2 μg, *Left*), the β_2 AR (2 μg, *Center*), or pcDNA3 (*Right*) alone or with either dynamin I Y231F/Y597F (7.5 μg) or β-arrestin 1 319–419 (7.5 μg). One day after transfection, cells were split into two 100-mm dishes and serum-starved overnight. After stimulation for 5 minutes with either 100 nM UK14304 (α_{2A} AR), 1 μM isoproterenol (β_2 AR), or 1 ng/ml EGF (EGFR), cell lysates were prepared, and the HA-ERK-1 was immunoprecipitated. Immunoblots were probed with both an anti-phospho-ERK 1/2 and a total ERK 1/2 antibody. Under each condition, data are expressed as the fold ERK 1/2 phosphorylation over the unstimulated. Data shown are the mean ± SEM of three independent experiments.

anti-HA affinity beads and rotated for 4 hr at 4°C, the immune complexes were washed twice with cold glycerol lysis buffer, denatured in 2× Laemmli sample buffer, and electrophoresed on SDS/PAGE gels. The proteins were transferred to poly(vinylidene difluoride) and probed for both phospho-ERK 1/2 and total ERK 1/2 as described below. Immunoprecipitation and detection of tyrosine phosphorylation of the EGFR was performed as described (16).

ERK 1/2 Phosphorylation. Serum-starved transfected cells grown in 12-well dishes were stimulated with agonist for 5 minutes at 37°C, the media aspirated, and the cells lysed in 100 μl of 2× Laemmli sample buffer. The samples were then electrophoresed on SDS/PAGE gels and transferred to poly(vinylidene difluoride). Phospho-ERK 1/2 was detected by using a 1:3,000 dilution of a rabbit polyclonal phospho-ERK 1/2-specific antibody (New England Biolabs), and total ERK 1/2 was detected by using a 1:1,000 dilution of an ERK 1/2 antibody (Upstate Biotechnology). Blots were probed with a 1:7,000 dilution of a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody. Blots were visualized by using ECL (enhanced chemiluminescence reagent; Amersham Pharmacia) and quantitated by using a scanning laser densitometer.

Immunofluorescence Microscopy. HEK-293 cells transiently expressing HA epitope-tagged α_{2A} ARs or β_2 ARs together with an EGFR–GFP fusion protein (23) were grown on sterile coverslips. Before stimulation, epitope-tagged receptors were labeled with a 1:100 dilution of a rhodamine-conjugated anti-HA antibody (Roche). Cells were then stimulated for 30 min at 37°C in the absence or presence of UK14304 (10 μM), isoproterenol (10 μM), or EGF (10 ng/ml) and fixed in 4% paraformaldehyde. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope by using a Zeiss 100× oil-immersion lens. Fluorescent signals were collected by using the Zeiss LSM software in the line switching mode by using dual excitation (488, 568 nm) and emission (515–540 nm, 590–610 nm) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

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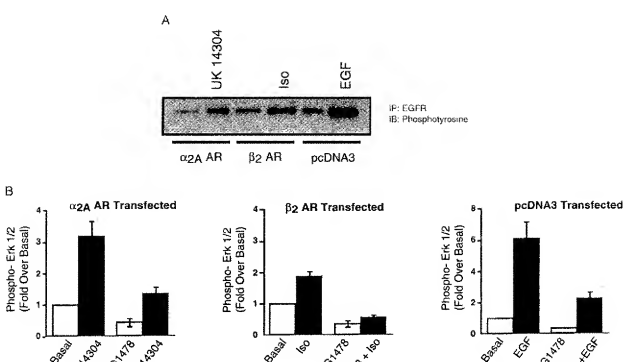

Fig. 3. UK14304, Isoproterenol- and EGF-stimulated tyrosine phosphorylation of the EGFR and the effect of the EGFR-specific tyrphostin, AG1478, on α_{2A} AR- and β_2 AR-mediated ERK 1/2 phosphorylation. (*A*) Serum-starved COS-7 cells transiently expressing the α_{2A} AR or β_2 AR or pCDNA3 were stimulated with 1 μM UK14304, 1 μM Isoproterenol, or 10 ng/ml EGF for 2 min. Monolayers were lysed in glycerol lysis buffer, and endogenous EGFRs were immunoprecipitated by using a sheep anti-human EGFR polyclonal antiserum. Immunoprecipitates were resolved by SDS/PAGE, and EGFR tyrosine phosphorylation was determined by immunoblotting by using a horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antiserum as described in *Materials and Methods*. (*B*) Cells transiently overexpressing the α_{2A} AR-, the β_2 AR-, or vector-transfected cells were preincubated for 15 min with tyrphostin AG1478 (125 nM) before stimulation with Isoproterenol (1 μM), UK14304 (1 μM), or EGF (10 ng/ml) for 5 min. ERK 1/2 phosphorylation was determined from whole-cell lysates as described in *Materials and Methods*. Data shown are the mean ± SEM of four independent experiments and are normalized to the level of ERK 1/2 phosphorylation in untreated cells.

Results and Discussion

As shown in Fig. 1*A*, when expressed in COS-7 cells, the β_2 AR undergoes agonist-driven internalization, whereas the α_{2A} AR does not. These data are consistent with previous studies that demonstrated that the α_{2A} AR exhibits little or no agonist-induced sequestration in either HEK293 (9, 24) or COS-1 (8) cells. In addition, as shown in Fig. 1*B*, activation of MAPK by both receptors is significantly dependent on the activation of pertussis toxin-sensitive G proteins.

Because both the α_{2A} AR- and the β_2 AR-mediated activation of ERK is pertussis toxin-sensitive, but the β_2 AR internalizes whereas the α_{2A} AR does not, this is an ideal system to examine the requirement for clathrin-mediated endocytosis in ERK activation. Our previous studies have suggested that for the β_2 AR, inhibitors of clathrin-mediated endocytosis block MAPK downstream of β_2 AR internalization (2). Moreover, in addition to a role for clathrin-mediated endocytosis in ERK activation by GPCRs, Vieira *et al.* (21) have suggested that clathrin-mediated endocytosis is involved in ERK activation mediated by the EGFR. Thus, we tested whether ERK activation by the α_{2A} AR, the β_2 AR, and the EGFR was sensitive to inhibitors of clathrin-mediated endocytosis. The effects of three mechanistically distinct inhibitors of clathrin-mediated endocytosis, MDC, Y231F/Y597F dynamin I, and β-arrestin 1 318–419, were determined. MDC inhibits clathrin-mediated endocytosis by stabilizing clathrin cages and has been shown to inhibit insulin-like growth factor-I (25) as well as LPA-mediated ERK activation (3). Y231F/Y597F dynamin I is a dominant inhibitory form of dynamin I that cannot be phosphorylated by c-Src (26), and β-arrestin 1 318–419 is a truncated form of β-arrestin 1 that interferes with GPCR sequestration through interactions with clathrin (27). MDC, Y231F/Y597F dynamin I, and β-arrestin 1 318–419 all inhibited agonist-stimulated internalization of the β_2 AR and the EGFR by 45–75% measured either by flow cytometry (β_2 AR) or by ^{125}I-labeled EGF-induced EGFR internalization (data not shown). As shown in Fig. 2*A*, MDC inhibited ERK 1/2 phosphorylation by the α_{2A} AR (*Left*), the β_2 AR (*Center*), and the EGFR (*Right*). Similarly, the dominant inhibitory forms of both dynamin I and of β-arrestin 1 significantly attenuated α_{2A} AR (Fig. 2*B*, *Left*), β_2 AR (Fig. 2*B*, *Center*) and EGFR-mediated (Fig. 2*B*, *Right*) MAPK activation. Thus, even though the α_{2A} AR itself does not internalize, the activation of MAPK by UK14304, like the activation by isoproterenol, is sensitive to all three inhibitors of clathrin-mediated endocytosis. Although consistent with a role for clathrin-mediated endocytosis in GPCR-mediated ERK activation, these data clearly dissociate sequestration of the GPCR from ERK activation. They also suggest that the G_i-dependent ERK signaling cascades used by the β_2 and α_{2A} ARs as well as the pathway used by

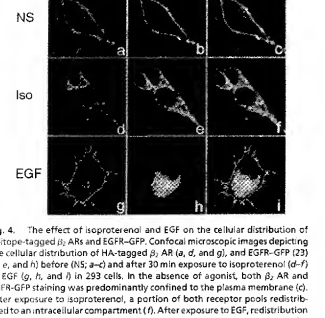

Fig. 4. The effect of isoproterenol and EGF on the cellular distribution of epitope-tagged β_2 ARs and EGFR–GFP. Confocal microscopic images depicting the cellular distribution of HA-tagged β_2 AR (*a*, *d*, and *g*), and EGFR–GFP (*23*) (*b*, *e*, and *h*) before (*NS*; *a–c*) and after 30 min exposure to isoproterenol (*d–f*) or EGF (*g*, *h*, and *i*) in 293 cells. In the absence of agonist, both β_2 AR and EGFR-GFP staining was predominantly confined to the plasma membrane (*c*). After exposure to isoproterenol, a portion of both receptor pools redistributed to an intracellular compartment (*f*). After exposure to EGF, redistribution of the EGFR–GFP, but not the β_2 AR, was observed (*i*). Qualitatively similar results have been obtained in COS-7 cells.

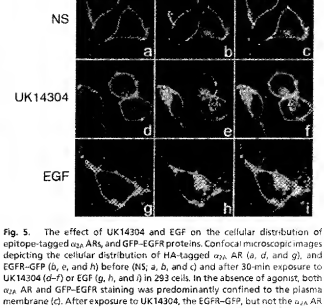

Fig. 5. The effect of UK14304 and EGF on the cellular distribution of epitope-tagged α_{2A} ARs, and GFP–EGFR proteins. Confocal microscopic images depicting the cellular distribution of HA-tagged α_{2A} AR (*a*, *d*, and *g*), and EGFR–GFP (*b*, *e*, and *h*) before (*NS*; *a*, *b*, and *c*) and after 30-min exposure to UK14304 (*d–f*) or EGF (*g*, *h*, and *i*) in 293 cells. In the absence of agonist, both α_{2A} AR and GFP–EGFR staining was predominantly confined to the plasma membrane (*c*). After exposure to UK14304, the EGFR–GFP, but not the α_{2A} AR redistributed to an intracellular compartment (*f*). A qualitatively similar pattern was observed after exposure to EGF, with redistribution of EGFR–GFP but not the α_{2A} AR (*i*). Qualitatively similar results have been obtained in COS-7 cells.

the EGFR in COS-7 cells all depend on clathrin-mediated endocytosis.

One pathway by which many GPCRs have been shown to activate ERK is via transactivation of RTKs, including the EGFR. For instance, the ET-1, LPA, and thrombin receptors in Rat-1 cells (*17*, *18*) the LPA (*20*), and the β_3 adrenergic receptors (*28*) in COS-7 cells each activate MAPK via transactivation of RTKs. To establish whether, in COS-7 cells, the α_{2A} AR- and β_2 AR-mediated activation of ERK 1/2 proceeds via a transactivation-dependent mechanism, we performed two experiments. First, we measured the ability of UK14304 and isoproterenol to stimulate increased tyrosine phosphorylation of the EGFR in cells expressing the α_{2A} AR or the β_2 AR. As previously reported for several GPCRs including the α_{2A} AR, the LPA receptor, and the thrombin receptor (*16*), UK14304, isoproterenol, and EGF stimulation each increases tyrosine phosphorylation of the EGFR (Fig. *3A*). Second, we measured the ability of tyrphostin AG1478, a selective EGFR inhibitor, to block α_{2A} AR-, β_2 AR- and EGFR-induced ERK 1/2 phosphorylation. As shown in Fig. *3B*, in appropriately transfected cells, tyrphostin AG1478 pretreatment attenuates the UK14304-, isoproterenol-, and EGF-induced ERK 1/2 phosphorylation. These data suggest that, in COS-7 cells, activation of the MAPK cascade by the α_{2A} AR, the β_2 AR, and the EGFR proceeds via a common mechanism, involving both clathrin-mediated endocytosis and activation of the EGFR.

Because transactivated EGFRs serve as an intermediate for α_{2A} AR and β_2 AR-mediated ERK activation, our data support the hypothesis that endocytosis of the EGFR or of another downstream effector accounts for the sensitivity of the GPCR signals to inhibitors of clathrin-mediated endocytosis. To examine whether stimulation of the α_{2A} AR and the β_2 AR leads to internalization of transactivated EGFRs, we used confocal immunofluorescence microscopy to examine the localization of each of these receptors after agonist treatment. Fig. 4 shows that in unstimulated cells transfected with the β_2 AR and the EGFR, both the β_2 AR and the EGFR localize primarily to the cell surface (*a–c*). Isoproterenol treatment of these cells leads to an increase in the intracellular localization of both the β_2 AR and the EGFR (*d–f*). EGF treatment of these cells, however, leads to an increase in EGFR localized inside the cells, whereas the β_2 AR remains on the cell surface (*g–i*). As shown in Fig. 5, treatment of cells expressing both the α_{2A} AR and the EGFR with either UK14304 (*d–f*) or EGF (*g–i*) leads to increased intracellular localization of the EGFR, whereas the α_{2A} AR remains localized on the cell surface. Thus, although activation of the β_2 AR leads to internalization of both the transactivated EGFR and the β_2 AR, UK14304 treatment of cells expressing the α_{2A} AR leads to internalization of the transactivated EGFR but not the α_{2A} AR. Our data suggest that GPCR-mediated transactivation of an RTK can lead to internalization of either the RTK alone (as is the case for the α_{2A} AR) or both the RTK and the GPCR (as is the case for the β_2 AR).

Recently Whistler and von Zastrow (*7*) reported that MAPK activation by the noninternalizing μ-opioid receptor is attenuated by a dominant inhibitory form of dynamin I. Their interpretation was that dynamin plays a unique signal transduction role distinct from its role in clathrin-mediated endocytosis. However, an alternative possibility is that the μ-opioid receptor, like the α_{2A} AR and the β_2 AR, mediates an endocytosis-dependent signal via EGFR transactivation. Our data, which indicate that several mechanistically distinct inhibitors of clathrin mediated endocytosis block GPCR-mediated ERK activation, are consistent with a more general role for the clathrin-mediated endocytic machinery in signal transduction.

RTK transactivation is but one mechanism of many by which GPCRs can activate the ERK cascade. We have previously demonstrated that the same GPCR can activate MAPK via multiple pathways and that the cellular context in which a receptor is expressed can determine the mechanism of GPCR-mediated MAPK activation (*20*). In addition to MAPK activation that

proceeds via the transactivation pathway, a second major pathway involves calcium and the tyrosine phosphorylation of the focal adhesion kinase (FAK)-like scaffolding protein, PYK2. Depending on the cell type, the contribution of transactivation (17) to ERK activation varies dramatically (20). In some cells, such as Rat-1 fibroblasts, the transactivation-dependent pathway is the major pathway to ERK activation, whereas in other cells such as PC12 cells, the PYK2 pathway is the major pathway. For instance, LPA receptor-mediated ERK activation can range from completely EGFR-dependent in Rat-1 cells to completely EGFR-independent in PC-12 cells (20). In HEK 293 cells, ERK 1/2 activation via both endogenous LPA receptor activation (20) and transiently expressed α_{2A} AR activation (data not shown) is only partially sensitive to tyrphostin AG1478. In these cells, the α_{2A} AR primarily activates ERK via a calcium-dependent signal that is blocked by a dominant-inhibitory mutant of the calcium-activated FAK family tyrosine kinase PYK2 (29). Such heterogeneity in GPCR signaling among cell types may account for the variable effects of clathrin-mediated inhibitors on MAPK that have recently been reported (2–14).

Taken together, our data suggest a model in which MAPK activation that proceeds via EGFR transactivation involves engagement of the clathrin-mediated endocytic machinery. What remains to be determined is whether endocytosis of a multiprotein complex including the EGFR and Raf is essential for transactivation-dependent MAPK activation or whether instead clathrin-coated endocytic pits serve some other function such as that of a specialized microdomain wherein signaling occurs.

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1. Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273**, 18677–18680.
2. Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G. & Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273**, 685–688.
3. Luttrell, L. M., Daaka, Y., Della Rocca, G. J. & Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 31648–31656.
4. Della Rocca, G. J., Mukhin, Y. V., Garnovskaya, M. N., Daaka, Y., Clark, G. J., Luttrell, L. M., Lefkowitz, R. J. & Raymond, J. R. (1999) *J. Biol. Chem.* **274**, 4749–4753.
5. Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K. H. & van Koppen, C. J. (1999) *J. Biol. Chem.* **274**, 12333–12338.
6. Ignatova, E. G., Belcheva, M. M., Bohn, L. M., Neuman, M. C. & Coscia, C. J. (1999) *J. Neurosci.* **19**, 56–63.
7. Whistler, J. L. & von Zastrow, M. (1998) *J. Biol. Chem.* **274**, 24575–24578.
8. DeGraff, J. L., Gagnon, A. W., Benovic, J. L. & Orsini, M. J. (1999) *J. Biol. Chem.* **274**, 11253–11259.
9. Schramm, N. L. & Limbird, L. E. (1999) *J. Biol. Chem.* **274**, 24935–24940.
10. Roche, J. P., Bounds, S., Brown, S. & Mackie, K. (1999) *Mol. Pharmacol.* **56**, 611–618.
11. Budd, D. C., Rae, A. & Tobin, A. B. (1999) *J. Biol. Chem.* **274**, 12355–12360.
12. Yang, W., Wang, D. & Richmond, A. (1999) *J. Biol. Chem.* **274**, 11328–11333.
13. Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L. & Liu-Chen, L. Y. (1999) *J. Biol. Chem.* **274**, 12087–12094.
14. Blanker, A., Pirard, A., Raijerson, R. M., Albene-Gelas, F., Muller-Esterl, W. & Dikic, I. (1999) *FEBS Lett.* **451**, 337–343.
15. Luttrell, L. M., Daaka, Y. & Lefkowitz, R. J. (1999) *Curr. Opin. Cell Biol.* **11**, 177–183.
16. Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K. & Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 4637–4644.
17. Daub, H., Weiss, F. U., Wallasch, C. & Ullrich, A. (1996) *Nature (London)* **379**, 557–560.
18. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. & Ullrich, A. (1997) *EMBO J.* **16**, 7032–7044.
19. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M. & Lefkowitz, R. J. (1995) *Nature (London)* **376**, 781–784.
20. Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J. & Luttrell, L. M. (1999) *J. Biol. Chem.* **274**, 13978–13984.
21. Vieira, A. V., Lamaze, C. & Schmid, S. L. (1996) *Science* **274**, 2086–2089.
22. Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. & Caron, M. G. (1994) *J. Biol. Chem.* **269**, 2790–2795.
23. Carter, R. E. & Sorkin, A. (1998) *J. Biol. Chem.* **273**, 35000–35007.
24. Daunt, D. A., Hurt, C., Hein, L., Kallio, J., Feng, F. & Kobilka, B. K. (1997) *Mol. Pharmacol.* **51**, 711–720.
25. Chow, J. C., Condorelli, G. & Smith, R. J. (1998) *J. Biol. Chem.* **273**, 4672–4680.
26. Ahn, S., Maudsley, S., Luttrell, L. M., Lefkowitz, R. J. & Daaka, Y. (1999) *J. Biol. Chem.* **274**, 1185–1188.
27. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H. & Benovic, J. L. (1997) *J. Biol. Chem.* **272**, 32507–32512.
28. Sneder, K. J., Sneddon, S. K., Cao, W. H., Della Rocca, G. J., Daniel, K. W., Luttrell, L. M. & Collins, S. (1999) *J. Biol. Chem.* **274**, 12017–12022.
29. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M. & Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 19125–19132.

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Communication

Targeted Construction of Phosphorylation-independent β-Arrestin Mutants with Constitutive Activity in Cells*

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Arrestin proteins play a key role in the desensitization of G protein-coupled receptors (GPCRs). Recently we proposed a molecular mechanism whereby arrestin preferentially binds to the activated and phosphorylated form of its cognate GPCR. To test the model, we introduced two different types of mutations into β-arrestin that were expected to disrupt two crucial elements that make β-arrestin binding to receptors phosphorylation-dependent. We found that two β-arrestin mutants (Arg^{169} → Glu and Asp^{383} → Ter) (Ter, stop codon) are indeed "constitutively active." *In vitro* these mutants bind to the agonist-activated β_2-adrenergic receptor (β_2AR) regardless of its phosphorylation status. When expressed in *Xenopus* oocytes these β-arrestin mutants effectively desensitize β_2AR in a phosphorylation-independent manner. Constitutively active β-arrestin mutants also effectively desensitize δ opioid receptor (DOR) and restore the agonist-induced desensitization of a truncated DOR lacking the critical G protein-coupled receptor kinase (GRK) phosphorylation sites. The kinetics of the desensitization induced by phosphorylation-independent mutants in the absence of receptor phosphorylation appears identical to that induced by wild type β-arrestin + GRK3. Either of the mutations could have occurred naturally and made receptor kinases redundant, raising the question of why a more complex two-step mechanism (receptor phosphorylation followed by arrestin binding) is universally used.

The decrease of a response to a persistent stimulus (desensitization) is a widespread biological phenomenon. Signaling by diverse G protein-coupled receptors (GPCRs)[1] is believed to be terminated by a uniform two-step mechanism (1). According to the model, activated receptor is first phosphorylated by a G protein-coupled receptor kinase (GRK). An arrestin protein binds to the activated phosphoreceptor, thereby blocking G protein interaction. Arrestin-receptor complex is then internalized, whereupon receptor is either dephosphorylated and recycled back to the plasma membrane (resensitization) or sorted to lysosomes and destroyed (down-regulation). Thus, the formation of the arrestin-receptor complex appears to be the final step of desensitization and the first step of resensitization and/or receptor down-regulation, which puts it at the crucial cross-roads of the processes regulating cellular responsiveness. The tremendously diverse superfamily of G protein-coupled receptors with more than 1000 members is the largest known group of proteins that translate a wide variety of external stimuli into intracellular "language." In contrast, the repertoire of receptor kinases and arrestins involved in the desensitization of these receptors is rather limited: only six GRKs and four arrestins have thus far been found in mammals (reviewed in Ref. 1). This suggests that at least some of the kinases and arrestins regulate numerous receptors. Thus, these proteins are attractive targets for research designed to delineate common molecular mechanisms underlying the regulation of GPCR signaling in cells (and to create fairly universal tools for the experimental and/or therapeutic intervention in the process).

EXPERIMENTAL PROCEDURES

Mutagenesis and Biochemical Characterization of β-Arrestins—Mutations Arg^{169} → Glu (CGG → GAG), Gln^{394} → Ter (CAA → TAA), and Asp383→Ter (GAT → TAG) were introduced by polymerase chain reaction in β-arrestin construct pHARR (3), that was used for *in vitro* transcription and translation, as described (3). NcoI/HindIII 1404-base pair open reading frame was then subcloned into appropriately digested *Escherichia coli* expression vector pTrcHisB (Invitrogen). All β-arrestin species were expressed in the *in vitro* translation system and tested in the direct binding assay (3), overexpressed in *E. coli*, purified to apparent homogeneity (16), and characterized in the agonist affinity shift assay (7), essentially as described.

Direct Binding Assay—*In vitro* translated tritiated arrestins (50 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol, 50 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin or with $P\text{-}\beta_2$AR or β_2AR (100 fmol/assay) in a final volume of 50 μl for 5 min at 30 °C in room light (rhodopsin) or for 60 min at 30 °C in the presence of 0.1 mM β-agonist isoproterenol. The samples were immediately cooled on ice and loaded onto 2 ml Sepharose 2B columns equilibrated with 20 mM Tris-HCl, pH 7.8, 2 mM EDTA. Bound arrestin eluted with receptor-containing membranes in the void volume (between 0.5 and 1.1 ml). Nonspecific binding determined in the presence of 0.3 μg of liposomes was subtracted.

Agonist Affinity Shift Assay—$P\text{-}\beta_2$AR or β_2AR (10–15 fmol/assay) was incubated in 0.25 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl (buffer A) containing 3 mg/ml bovine serum albumin in the presence of 65–75 fmol of ^{125}I-iodopindolol (NEN Life Science Products) and the indicated concentrations of arrestins and agonists for 60 min at 22 °C. Samples were then cooled on ice and loaded at 4 °C onto 2 ml of Sephadex G-50 columns. Receptor-containing liposomes with bound radioligand were eluted with buffer A (between 0.6 and 1.5 ml), and radioactivity was quantitated in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM alprenolol. All experiments were repeated two to three times, and data are presented as means ± S.D.

Desensitization Studies in Xenopus Oocytes—Stage IV oocytes from

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; βARK2, β-adrenergic receptor kinase 2 (GRK5); Kir3.4, protein-gated inwardly rectifying K$^+$ channel; β_2AR, β_2-adrenergic receptor; $P\text{-}\beta_2$AR, phosphorylated β_2AR; DOR, δ opioid receptor; Rh*, light-activated rhodopsin; P-Rh*, phosphorylated Rh*; HAC, high agonist affinity complex; Ter, stop codon.

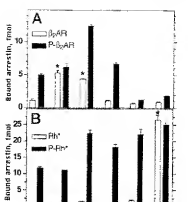

FIG. 1. **Direct binding assay.** *A*, 100 fmol of βARK-phosphorylated (*P-*β_2*AR*, 2.7 ± 0.2 mol phosphate/mol receptor) or unphosphorylated purified β_2AR reconstituted into liposomes was incubated in a 50 μl reaction with 50 fmol of the indicated form of tritiated arrestin (specific activities: 140–160 dpm/fmol) in the presence of 100 μM agonist isoproterenol in 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM $MgCl_2$, for 45 min at 30 °C. *B*, 0.3 μg of rhodopsin kinase-phosphorylated (*P-Rh**, 1.6 ± 0.1 mol of phosphate/mol) or unphosphorylated (*Rh**) rhodopsin was incubated with the same set of arrestins under room light for 5 min at 37 °C. The samples were then cooled on ice and loaded at 4 °C onto 2-ml Sepharose 2B columns, equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA. Bound tritiated arrestins were eluted with receptor-containing membranes in the void volume (between 0.5 and 1.1 ml), and the radioactivity was quantitated in a liquid scintillation counter. *, $p < 0.01$, Student's *t* test, compared with the binding of corresponding wild type arrestin.

mature female *Xenopus laevis* frogs were harvested, defolliculated, and cultured as described previously (8). cRNA was prepared for oocyte injection from cDNA template using Ambion message machine kit (Ambion, TX) according to manufacturer's protocol. cDNAs (GenBank[TM] accession numbers in parentheses) for rat GRK3 (AA144583), human β_2AR (AJ052644), mouse δ-opioid receptor (L06322), and rat G protein-gated inwardly rectifying potassium channel subunits Kir3.1 (U01071) and Kir3.4 (X83584) were amplified and linearized prior to cRNA synthesis. cDNAs for all forms of β-arrestin were first amplified by polymerase chain reaction using oligonucleotides designed to add a T7 promoter upstream and a 45-base poly(A) tail downstream of β-arrestin open reading frame. Standard two-electrode voltage clamp recordings were performed to register Kir3 currents activated by agonist perfusion as described (8). The expression levels in oocytes of all forms of β-arrestin were determined by quantitative Western blot with F4C1 anti-arrestin antibody (22), as described (16). Means ± S.D. from four to six measurements are presented.

RESULTS AND DISCUSSION

Recently we have proposed a molecular mechanism that explains an amazing selectivity of arrestins for the activated phosphorylated forms of GPCRs (2, 3). According to previous *in vitro* studies (2, 3) arrestins have two primary binding sites: an activation-recognition site that recognizes the agonist-activated state of the receptor and a phosphorylation-recognition site that interacts with GRK-phosphorylated elements of the receptor. A potent secondary receptor-binding site is mobilized for the interaction only when both primary sites are simultaneously engaged, *i.e.* when an arrestin encounters activated and phosphorylated receptor (2, 3). In this model, arrestin is kept in its basal conformation by several intramolecular interactions in which certain residues in the primary binding sites ("trigger" residues) are involved. One of these triggers is pulled by binding to an activated form of the receptor, the other, by the interaction of arrestin with phosphate(s) introduced by GRK. Thus, arrestin works like a coincidence detector, assuming its high-affinity receptor-binding conformation when both triggers are simultaneously pulled. The model of sequential

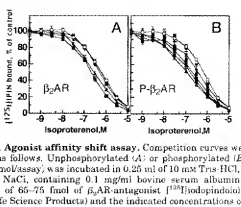

FIG. 2. **Agonist affinity shift assay.** Competition curves were generated, as follows. Unphosphorylated (*A*) or phosphorylated (β_2AR (10–15 fmol/assay) was incubated in 0.25 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, containing 0.1 mg/ml bovine serum albumin in the presence of 65–75 fmol of β_2AR-antagonist, [^{125}I]iodopindolol (*IPIN*) (NEN Life Science Products) and the indicated concentrations of β_2AR-agonist isoproterenol for 60 min at 22 °C in the absence (○) or presence of 1 μM β-arrestin (●), β-arrestin-1-Arg^{169} → Glu (▲), β-arrestin-(1–382) (■), or β-arrestin-(1–393) (♦). Samples were then cooled on ice and loaded onto 2-ml Sephadex G-50 columns at 4 °C. Receptor-containing liposomes with bound radioligand were eluted with the same buffer (between 0.6 and 1.5 ml), and the radioactivity was quantitated. For the unphosphorylated β_2AR competition curves in the absence of arrestins and in the presence of wild type and β-arrestin (1–393) are monophasic (analysis using Prism 2.0 for Power Macintosh). In the presence of β-arrestin-(Arg^{169} → Glu) and β-arrestin-(1–382) the curves are biphasic, suggesting the presence of high- and low-affinity sites. With P-β_2AR competition curves generated in the presence of all forms of β-arrestin are biphasic, while the curve in the absence of arrestins is monophasic. The analysis shows that in all cases isoproterenol competes for the binding at high-affinity sites with IC_{50} of 26 ± 6 nM and the low-affinity sites with IC_{50} of 730 ± 131 nM. The latter is very close to the affinity of β_2AR alone (656 ± 27 nM). The fraction of the high-affinity binding sites in each experiment is presented in the text. Experiments were performed two to three times in duplicates. Means ± S.D. are presented.

multisite binding (2, 3), and the recent crystal structure of visual arrestin (4) set the stage for the targeted construction of arrestin mutants in which one of the triggers is constitutively pulled by an appropriate mutation.

In order to test the validity of the model we constructed three β-arrestin mutants: 1) Arg^{169} → Glu, that reverses the charge of the putative phosphorylation-sensitive trigger (Arg^{169} → Glu is homologous to the Arg^{175} → Glu mutation in visual arrestin, that makes its binding to rhodopsin phosphorylation-independent (5)); 2) Gln^{394} → Ter; and 3) Asp^{395} → Ter, that delete a part or all of the regulatory arrestin COOH terminus, which keeps arrestin in a basal conformation and suppresses an untimely mobilization of the secondary binding site (6).

First, we tested the ability of these mutants to interact with purified β_2AR reconstituted into phospholipid vesicles by performing direct binding studies (3) and agonist affinity shift assays (7) *in vitro*. Wild type β-arrestin and β-arrestin-(1–393) bind poorly to activated unphosphorylated receptor (Fig. 1*A*). In contrast, β-arrestin-(Arg^{169} → Glu) and β-arrestin-(1–382) demonstrate significantly higher binding to activated unphosphorylated receptor (Fig. 1*A*). Wild type β-arrestin and all three mutants readily bind to activated and phosphorylated β_2AR (Fig. 1*A*). Thus, β-arrestin-(Arg^{169} → Glu) and β-arrestin-(1–382) bind to activated β_2AR in a phosphorylation-independent fashion.

Recently (7) we found that arrestin-receptor complex is similar to G protein-receptor complex in two respects. Agonists have higher affinity for arrestin-receptor complex than for receptor alone, and only a fraction of the receptors forms such a high agonist affinity complex (HAC) even at saturating concentrations of arrestin. The maximum percentage of the receptor in HAC gives a good estimate of the propensity of a given arrestin protein to bind tightly to the receptor (arrestin *competency*) (7). Consistent with the direct binding data (Fig. 1*A*),

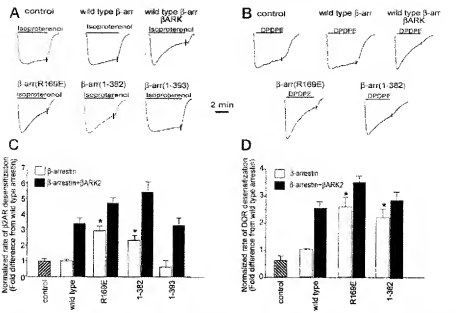

FIG. 3. **GRK-independent functional desensitization of β_2AR and DOR by β-arrestin-($Arg^{169} \rightarrow Glu$) and β-arrestin-(1–382).** *A* and *C*, β_2AR function was studied in oocytes injected with a mixture of cRNAs for the β_2AR, the G protein-gated inwardly rectifying K^+ channel subunits Kir3.1 and Kir3.4, and $G_s\alpha$, that allows the G_s-coupled β_2AR to activate the coexpressed channels, as described (8). *B* and *D*, DOR was studied in oocytes injected with cRNA for DOR, Kir3.1, and Kir3.4 (8). As indicated, some oocytes were also coinjected with 8 ng of cRNA for the different forms of β-arrestin either alone or together with 0.5 ng of βARK2 cRNA. All recordings were performed 3–4 days post-injection. Receptor-activated currents were measured in 16 mM K^+ buffer (8) at −80 mV holding potential. The agonist-elicited responses were adjusted by base-line subtraction as described (8) and normalized to the peak response. The *short vertical lines* through the traces indicate when agonist treatment was discontinued and the corresponding antagonist perfusion started. Antagonist perfusion was used to determine the amount of residual receptor response. Calibration scales are the same for each trace (2 min). Representative traces depicting β_2AR-activated current responses elicited by 1 μM β-agonist isoproterenol and reversed by 1 μM of β-antagonist propranolol (*A*), DOR-activated current responses elicited by 1 μM DOR agonist, DPDPE and reversed by 1 μM DOR antagonist naloxone (*B*), the β_2AR desensitization rate in each group of oocytes expressed as a multiple of the desensitization rate in oocyte group injected with cRNA for wild type β-arrestin only (*C*), and DOR desensitization rates calculated as for β_2AR (*D*) are shown. *Hatched bars* in *C* and *D* represent the corresponding control receptor desensitization rates measured in oocytes expressing neither βARK2 nor β-arrestin. *, $p \leq 0.05$, Student's *t* test, compared with desensitization rate of the group coexpressing wild type β-arrestin only. Each *bar* represents the mean ± S.E. from 4–16 separate oocytes. *β-arr*, β arrestin.

β-arrestin-($Arg^{169} \rightarrow Glu$) and β-arrestin-(1–382) induced the formation of HAC by unphosphorylated β_2AR (22 ± 4% in both cases) (Fig. 2*A*). In contrast, all forms of β-arrestin induced the formation of HAC by phosphorylated β_2AR (P-β_2AR) (Fig. 2*B*). The percentage of HAC formed by P-β_2AR in the presence of saturating (1 μM) concentration of β-arrestin, β-arrestin-($Arg^{169} \rightarrow Glu$), β-arrestin-(1–382), and β-arrestin-(1–393) was 31 ± 6, 52 ± 3, 41 ± 3, and 20 ± 4%, respectively (Fig. 2*B*). In summary, in both *in vitro* assays β-arrestin-($Arg^{169} \rightarrow Glu$) and β-arrestin-(1–382) demonstrate constitutive activity (phosphorylation-independent receptor binding).

Next we tested whether these β-arrestin species can functionally desensitize unphosphorylated β_2AR in living cells. To this end GPCRs were expressed in *Xenopus* oocytes and the activation of coexpressed G protein-gated inwardly rectifying K^+ channel Kir3 was used as a measure of receptor function. Under these conditions, the application of receptor agonists produced a large increase in inwardly rectifying potassium conductance (8). Undetectable levels of endogenous arrestins and GRKs are expressed in these cells (data not shown). As a result, only a very slow response desensitization was evident during prolonged agonist treatment when β_2AR (or another GPCR) is expressed alone. The rate of desensitization was not significantly increased when the receptor is coexpressed with either GRK alone or arrestin alone. However, a dramatic increase in desensitization rate was observed when both GRK and arrestin were coexpressed with a receptor (8, 9). To compare the relative activity of different forms of β-arrestin, we expressed β_2AR with or without GRK3 (also called β-adrenergic receptor kinase 2 or βARK2) in the presence or absence of different forms of β-arrestin. As shown on Fig. 3, *A* and *C*, both wild type and β-arrestin-(1–393) facilitated β_2AR desensitization only when βARK2 was present. In contrast, β-arrestin-($Arg^{169} \rightarrow Glu$) and β-arrestin-(1–382) in the absence of βARK2 produced high rates of desensitization similar to that produced by wild type β-arrestin in the presence of βARK2, suggesting that these mutants do induce phosphorylation-independent desensitization of β_2AR in the cell.

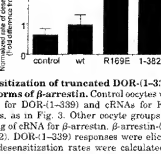

FIG. 4. **Desensitization of truncated DOR-(1–339) by "constitutively active" forms of β-arrestin.** Control oocytes were injected with 0.4 ng of cRNA for DOR-(1–339) and cRNAs for Kir3.1 and Kir3.4 channel subunits, as in Fig. 3. Other oocyte groups were in addition injected with 8 ng of cRNA for β-arrestin, β-arrestin-($Arg^{169} \rightarrow Glu$), or β-arrestin-(1–382). DOR-(1–339) responses were elicited by 1 μM DPDPE. Receptor desensitization rates were calculated as described in Fig. 3 and normalized to the response desensitization rate in the oocyte group expressing wild type β-arrestin. *, $p < 0.05$, Student's *t* test, compared with desensitization rate of the group with wild type β-arrestin.

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In order to test whether the constitutively active forms of β-arrestin retain the characteristic broad receptor specificity of wild type nonvisual arrestins (9, 10), we performed similar series of experiments with δ opioid receptor (DOR) (Fig. 3, *B* and *D*), which was previously shown to be desensitized following agonist activation in oocytes coexpressing wild type β-arrestin and βARK2 (8). Again, the constitutively active mutants induced DOR desensitization, even in the absence of βARK2, suggesting that these mutations do not appreciably change receptor specificity of β-arrestin (or, rather, lack thereof). It should be noted that wild type visual and β-arrestin, visual arrestin mutant ($Arg^{175} \rightarrow Glu$), and β-arrestin mutants ($Arg^{169} \rightarrow Glu$), (1–382), and (1–393), readily bind to activated phosphorylated forms of both rhodopsin and β_2AR (Fig. 1). Visual arrestin mutant ($Arg^{175} \rightarrow Glu$) also binds to unphosphorylated activated rhodopsin, while β-arrestin mutants ($Arg^{169} \rightarrow Glu$) and (1–382) bind to unphosphorylated activated β_2AR. However, phosphorylation-independent visual arrestin mutant does not bind to unphosphorylated activated β_2AR and phosphorylation-independent β-arrestin mutants do not bind to unphosphorylated activated rhodopsin (Fig. 1). Thus, the preference of β-arrestin for β_2AR over rhodopsin and that of visual arrestin for rhodopsin over β_2AR (3) appears, if anything, enhanced by these mutations.

Interestingly, in the presence of βARK2 both of the phosphorylation-independent β-arrestin mutants induced a more rapid receptor desensitization than wild type β-arrestin (Fig. 3), although the expression levels of all forms of β-arrestin in oocytes were virtually the same (0.72 ± 0.34, 0.90 ± 0.27, 0.85 ± 0.32, and 1.44 ± 0.87 ng/μg of total protein for wild type, ($Arg^{169} \rightarrow$ Glu), (1–382), and (1–393) forms, respectively). Apparently, faster desensitization in the presence of βARK2 reflects stronger binding of the mutants to phosphorylated receptor (Figs. 1, 2). Because the peak agonist-induced β_2AR and DOR responses were not significantly different in oocytes expressing constitutively active β-arrestins (compared with oocytes expressing no β-arrestin or wild type β-arrestin; data not shown), the mutants do not appear to be prebound to the receptor before agonist application.

Our previous studies demonstrated that the crucial GRK phosphorylation sites are localized on the carboxyl-terminal part of DOR, and that the truncation of the receptor (DOR-(1–339)) blocked homologous desensitization mediated by β-arrestin + βARK2 (8). We tested whether constitutively active β-arrestin mutants can rescue the desensitization of DOR-(1–339). Both phosphorylation-independent β-arrestin mutants induced the desensitization of truncated DOR with virtually the same kinetics as evident for the full-length DOR (Fig. 4). These data suggest that constitutively active β-arrestins are equally capable of tight binding to (and blocking the signaling of) a receptor without phosphates on the COOH terminus and without the COOH terminus itself. An important implication of this finding is that the major role of the GRK-phosphorylated elements of the receptor is to pull the phosphorylation-sensitive trigger on the arrestin molecule; they do not appear to be required for tight arrestin binding *per se*.

Thus, the binding of β-arrestin-($Arg^{169} \rightarrow$ Glu) and β-arrestin-(1–382) to unphosphorylated receptor detectable in both *in vitro* assays (Figs. 1 and 2) translates into the ability of these mutants to induce phosphorylation-independent receptor desensitization in the living cell (Figs. 3 and 4). Taken together, the data corroborate the model of sequential multisite arrestin-receptor interaction (2, 3) and open an enticing prospect of targeted construction of mutant arrestins with different special functional characteristics. Recent studies suggest that arrestin binding targets the receptors for internalization (10, 11), apparently by virtue of the ability of nonvisual arrestins to interact with clathrin (12), which is unaffected by the mutations introduced in this study (data not shown). β-Arrestin mutants capable of tight phosphorylation-independent binding to the receptor may change the pattern of intracellular receptor trafficking.

Phosphorylation-independent arrestins are likely to prove valuable tools for the experimental manipulation of the efficiency of signaling by different GPCRs. Uncontrolled signaling by various naturally occurring mutant forms of G protein-coupled receptors has been linked to a wide variety of pathological conditions in humans, from stationary night blindness and retinitis pigmentosa (Refs. 13 and 14 and references therein) to Jansen-type metaphyseal chondrodysplasia (15), autosomal dominant hypocalcemia (17, 18), autosomal dominant hyperthyroidism (19, 20), and numerous forms of cancer (reviewed in Ref. 21). Arrestin mutants with an enhanced ability to block this excessive signaling appear promising tools for the gene therapy of these disorders.

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REFERENCES

1. Freedman, N. J., and Lefkowitz, R. J. (1996) *Recent Prog. Horm. Res.* **51**, 319–351
2. Gurevich, V. V., and Benovic, J. L. (1993) *J. Biol. Chem.* **268**, 11628–11638
3. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. (1995) *J. Biol. Chem.* **270**, 720–731
4. Granzin, J., Wilden, U., Choe, H.-W., Labahn, J., Krafft, B., and Buldt, G. (1998) *Nature* **391**, 918–921
5. Gurevich, V. V., and Benovic, J. L. (1995) *J. Biol. Chem.* **270**, 6010–6016
6. Gurevich, V. V. (1996) *J. Biol. Chem.* **271**, 15501–15506
7. Gurevich, V. V., Pals-Rylaarsdam, R., Benovic, J. L., Hosey, M. M., and Onorato, J. J. (1997) *J. Biol. Chem.* **272**, 28849–28857
8. Kovoor, A., Nappey, V., Kieffer, B. L., and Chavkin, C. (1997) *J. Biol. Chem.* **272**, 27603–27611
9. Kovoor, A., Celver, J. P., Wu, A., and Chavkin, C. (1998) *Mol. Pharmacol.* **54**, 704–711
10. Barak, L. S., Ferguson, S. S. G., Zhang, J., and Caron, M. G. (1997) *J. Biol. Chem.* **272**, 27497–27500
11. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271**, 363–366
12. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**, 447–450
13. Rao, V. R., Cohen, G. B., and Oprian, D. D. (1994) *Nature* **367**, 639–642
14. Chen, J., Makino, C. L., Peachey, N. S., Baylor, D. A., and Simon, M. I. (1995) *Science* **267**, 374–377
15. Schipani, E., Kruse, K., and Jüppner, H. (1995) *Science* **268**, 98–100
16. Grey-Keller, M. P., Detwiler, P. B., Bonvini, E. J., and Gurevich, V. V. (1997) *Biochemistry* **36**, 7058–7063
17. Rao, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S., Pollak, M., Krapcho, K., Hebert, S., and Brown, E. M. (1996) *J. Biol. Chem.* **271**, 14537–14540
18. Pearce, S., Trump, D., Wooding, C., Besser, G., Chew, S., Heath, D., Hughes, I., and Thakker, R. (1995) *J. Clin. Invest.* **52**, 726–740
19. Duprez, L., Parma, J., Van Sande, J., Allgeier, A., Leclere, J., Schvartz, C., Delisle, M. J., Decoulx, M., Orgiazzi, J., Dumont, J. E., and Vassart, G. (1994) *Nat. Genet.* **7**, 396–401
20. Peschke, B., and Ludgate, M. (1997) *N. Engl. J. Med.* **337**, 1675–1681
21. Gutkind, J. S. (1998) *J. Biol. Chem.* **273**, 1839–1842
22. Donoso, L. A., Gregerson, D. S., Smith, L., Robertson, S., Kissane, V., Vrabec, T., and Salnow, C. M. (1990) *Curr. Eye Res.* **9**, 345–353